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14. ABSTRACT Recent data indicate that castration-recurrent prostate cancer (CR-CaP) progression is driven by the activation of wild-type androgen receptor (AR) through at least two mechanisms: tyrosine phosphorylation by Src family (SFK) and Ack1 tyrosine kinases, and the induction of AR coregulators that regulate the transcriptional activity of AR. It is likely that identifying novel AR-regulated genes in CR-CaP, especially those involving promoters with novel target sequences, will help elucidate the molecular mechanisms that drive CR-CaP initiation and progression, and will help identify potential new therapeutic targets for CR-CaP. We propose to use ChIP-seq, exome-seq and bioinformatics analyses to comprehensively identify AR-regulated genes that drive the growth of human CR-CaP tumors in mice. The long-term aim of this work is to develop a CR-CaP progression gene signature, to identify CR-CaP-associated AR binding site motifs, and to identify potential new therapeutic targets in CR-CaP.					
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INTRODUCTION

This study seeks to identify the androgen receptor (AR) cistrome associated with castration-recurrent prostate cancer (CR-CaP) based on *in vivo* growth conditions. Our hypothesis is that modification of AR in CR-CaP, including direct tyrosine phosphorylation by Src family tyrosine kinases (SFK) and the Ack1 tyrosine kinase, alters its interaction with transcriptional cofactors, resulting in transcriptional regulation of pro-malignancy genes through the recognition of both androgen response elements (ARE) and novel gene promoter sequences. The cistrome will be identified by ChIP-Seq from tumor lysates of human CaP grown in nude mice under androgen-dependent (AD) and castrate conditions, and in parallel, RNA from these samples will be analyzed for changes in gene expression. A subset of promoter sequences engaged by AR under AD and androgen-independent (AI) conditions will be validated by ChIP assay. Finally, the data will be analyzed through bioinformatics programs that predict how the AD and AI regulated genes contribute to CR-CaP progression and growth.

BODY

Below are each of the tasks approved in the Statement of Work followed by a report on how the tasks aims were met (or not), or how experiments were adapted to address the intent of the tasks (in bold)

Task 1. Transduce CaP cells with CA-Src or –Ack 1: Produce tet-regulated lentiviruses (pLVTH) with CA-Src or –Ack1 (or empty vector), infect LNCaP –and LAPC-4-luciferase cells, select for puromycin-resistant cells, verify tet-inducible expression of CA-kinases using phosphor-specific Abs in immunoblots.

Progress- We produced stable LNCaP-luciferase cells expressing CA-Src or –Ack1 plus either WT-, Y267F- or Y534F-AR (Fig. 1). These cells were validated for expression of exogenous Src, Ack1 or AR proteins by immunoblot (IB). LAPC-4 cells have been transfected with the constructs described above and puromycin-resistant colonies selected. Clones will be isolated and validated for the transduced proteins.

Task 2. Isolate and analyze CaP cell lysates and RNA regulated by androgens and CA-kinase induction: Grow the CaP cell lines from Task 1 under growth conditions: vehicle vs. R1881, + or – tet. Perform AR-specific ChIP-Seq and transcriptome analysis.

Progress- In order to demonstrate that the LNCaP cell lines retained i) appropriate androgen inducibility of AR-regulated genes, the LNCaP-luc control cells were treated for various times with 1 nM dihydrotestosterone (DHT) and the transcription level of two inducible genes, *TMPRSS2* and *PSA*, as well as AR itself, were assessed by qRT-PCR (Fig. 2A). Note that we switched to using DHT instead of synthetic testosterone (R1881) because we got responses at more physiologic androgens levels. As published by many other groups, *TMPRSS2* and *PSA* expression was increased by DHT whereas AR transcription was suppressed. Whereas the PSA promoter androgen-response element (ARE) shows high levels of RNA Polymerase II (Pol II) binding in the absence of DHT (Fig. 2B, lower panel) as shown by chromatin immunoprecipitation (ChIP), which is nonetheless induced ~2-fold by DHT treatment (1 nM for 16h), the so-called PSA enhancer ARE shows significant Pol II binding only upon DHT treatment (Fig. 2B, upper panel). Importantly, expression of CA-Src induces androgen-independent activation of AR genes such as PSA using the enhancer ARE in AR-ChIP assays (Fig. 2C). Similar results were shown using the enhancer ARE from *TMPRSS2* (not shown). Taken together, these data indicate that the LNCaP/CA-Src cells show androgen-independent AR-specific gene regulation akin to CR-CaP. We are in the process of completing similar analyses using LNCaP/CA-Ack1 cells, as well as CA-Src cells expressing WT-, Y267F- or Y534F-AR. We expect that the AI activation of AR will be abrogated in cells overexpressing the Y→F mutant appropriate for their CA-kinase (Y267 for Ack1,

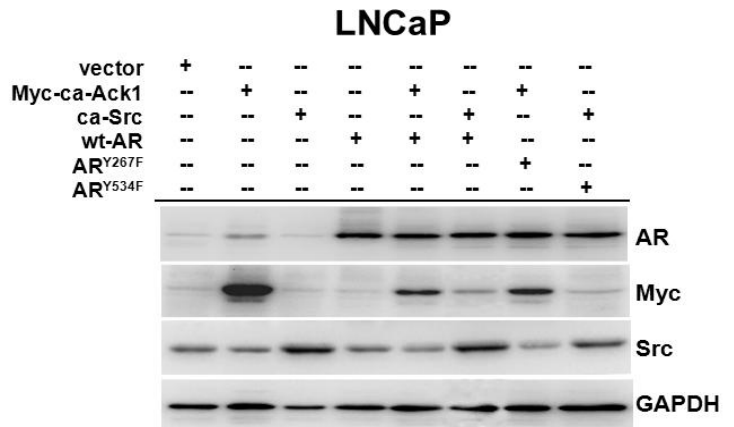


Fig. 1. Stable LNCaP-luc cell lines expressing CA-Src or –Ack1 plus WT, Y267F or Y534F AR. Cell lysates were probed by immunoblot (IB) for AR, myc-tagged Ack1, Src or GAPDH (protein loading control).

Y534 for Src). We will follow up these analyses in the LAPC-4 lines currently in development.

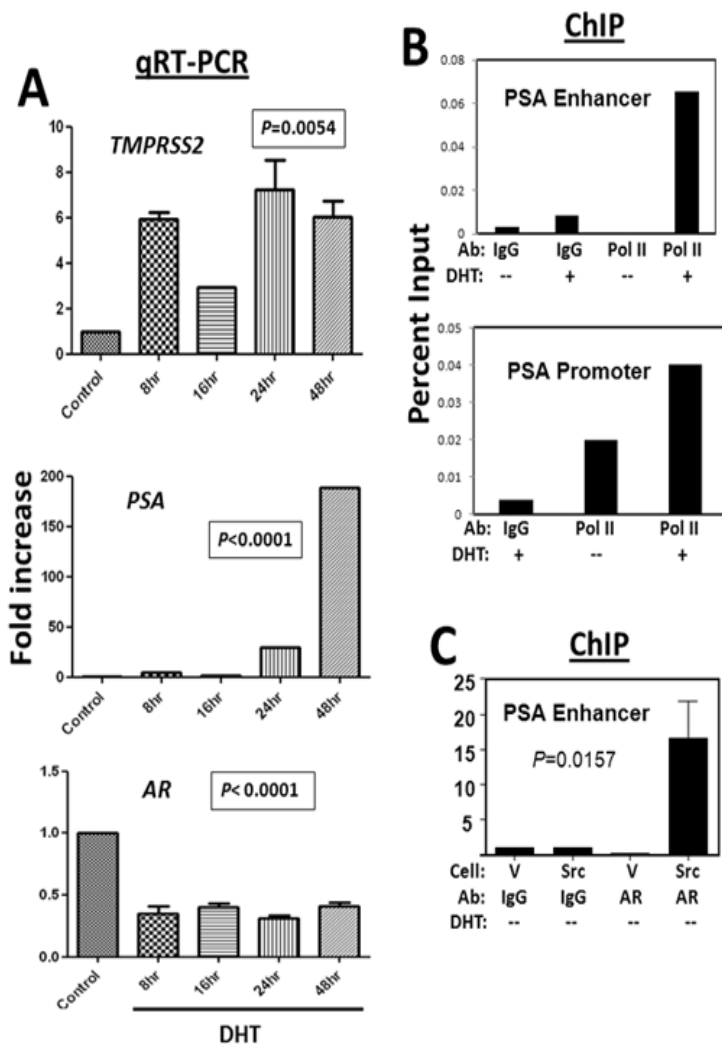


Fig. 2. CA-Src induces AI activation of AR. (A) qRT-PCR assays for transcript levels of *TMPRSS2*, *PSA*, and *AR* in LNCaP-luc treated with DHT (1 nM) for various times periods. ChIP analysis of the PSA enhancer or promoter ARE after IP with IgG or anti-Pol II (Panel B), or IgG or anti-AR (Panel C) in LNCaP-luc (Panel B) or LNCaP-luc cells transduced with vector (V) or CA-Src (Panel C).

remaining 15 mice, remove T-pellets and immediately start feeding tet-drinking water. Sacrifice mice (5/time point) 1, 3 and 5 days later, isolate cell lysates and RNA, perform AR-specific ChIP-Seq and transcriptome analysis on all samples.

Progress- We have produced tumors in androgen-supplemented mice (some of which were isolated as AD), and after castration, only the LNCaP/luc tumors transduced with either CA-Src or -Ack1 progressed; the vector cells did not regress but rather stopped growing (data not shown). Tumors from all these conditions were isolated. RNA has been isolated from portions and other parts will be minced in order to perform AR-ChIP. These samples have been converted into next-gen libraries, sequenced and analyzed as above.

Task 4. Analyze and correlate ChIP-Seq and transcriptome data to identify AD-and AI-AR-

In order to perform AR-ChIP-seq, we first did large scale AR-ChIP on LNCaP-luc/vector or /CA-Src cells grown in androgen-depleted vs. DHT-treated (16h, 1 nM) conditions, and then sheared the resulting DNA using a Covaris disruptor. Our fragment sizes were ~100-300bp, which is ideal for ChIP-seq since these are roughly nucleosome unit-sized and also appropriate for the shorter reads of the Illumina HiSeq2000 platform flow cells.

Next-gen libraries were prepared from the following samples, which were then sequenced in our Illumina platform: LNCaP-luc/V, /CA-Src, /CA-Ack1, /CA-Src+WT-AR and /CA-Src+AR-Y534F, either androgen-starved or DHT-treated. In parallel, RNA from these samples was converted into cDNA and then into next-gen libraries for transcriptome-seq analyses. We are currently analyzing the ChIP-seq read frequencies and mapping them to chromosomal locations using Genomatrix software such as RegionMiner. Once the transcriptome data are produced, we will show how Src- and/or DHT-induced changes to AR binding sites affects gene expression, with the assumption that genes engaged and differentially regulated by AR in CA-Src cells under AI conditions might likely represent genes that drive CR-CaP. The analyses with the Ack1 cell group has been completed in the last year's NCE.

Task 3. Isolate and analyze CaP tumor lysates and RNA regulated by androgens and CA-kinase induction: Orthotopically inject castrated, T-pelleted male nude mice (20 mice/group) with 10^6 CaP-

luciferase/CA-Src or -Ack1, or empty vector, grow tumor to ~500mm³ (monitored by weekly IVIS: i.p. inject mice with luciferin, and then bioimage tumor growth after 1h in anesthetized mice). Sacrifice 5 mice, isolate cell lysates and RNA. Of the

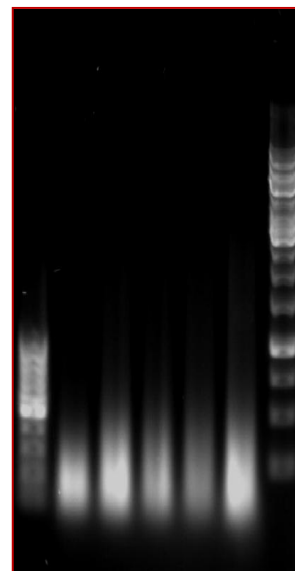


Fig. 3. Appropriate DNA fragment sizes after shearing, compared to 100bp and 1Kb marker ladders on the left and right lanes, respectively.

regulated genes: Perform signal-to-noise filtering of data including AR-regulated gene identity and relative abundance, correlate genes identified by ChIP-Seq with similar changes in transcriptome analyses using ANOVA and other statistical packages. Develop a core signature of 8-15 of the strongest AI-AR-regulated genes.

Progress- We have generated three major Src- and Ack1-regulated genes signature groups we believe impact the formation of CR-CaP: 1) Src/Ack-regulated genes that are AR dependent and that are regulated in a similar fashion by DHT in AD cells such as LNCaP or VCaP, 2) Src/Ack-regulated genes that are regulated in an opposite fashion by AR, 3) genes regulated by Src and/or Ack1 that are AR-independent. With the recent publication of an AR-ChIP-seq and transcriptome analysis of human androgen sensitive vs. CR-CaP samples by Sharma et al., Cancer Cell, 2013, 23(1):35-47), we have endeavored to compare our signatures to those of CR-CaP patients by comparative bioinformatics analyses.

Task 5. Validate subset of putative AI-AR-regulated genes: Perform direct ChIP analyses, qRT-PCT, and web-based in silico analyses (Oncomine and GEO) on 8-15 AI-AR-regulated genes identified by Task 4. Perform IHC on CaP TMAs, correlate increased or decreased staining with clinical/pathological status (pre- or post-castration, primary vs. CR-CaP, time from castration to CR-CaP, serum PSA levels, Gleason sum, metastasis, survival). If appropriate Abs can be readily identified, this IHC analysis could include as many as 8-15 protein products of the AI-AR gene signature.

Progress- Completed (yet ongoing). We have performed AR-, H3K27me3 and Pol-II-ChIP, and qRT-PCR analyses of at least 20 genes falling into one of three signatures (above) for AR promoter/enhancer occupancy in AD- vs. CR-CaP cells lines, +/- DHT or AR-siRNA (vs. scrambled siRNA). All the identified genes have been validated in this way, indicating that our screen and bioinformatics analyses were appropriate to identify Src/Ack1-regulated genes potentially contributing to CR-CaP. The TMA staining has yet to be completed. However, we are correlating our gene signature lists to databanks such as those in ENCODE and *cbio* in order to better correlate the changes with CR-CaP and/or CaP metastasis.

Task 6. Prepare report. (Month 12/24). Completed here.

KEY RESEARCH ACCOMPLISHMENTS

- successful transduced LNCaP and LAPC-4 CaP cells with CA-Src, CA-Ack1, WT- and Y→F-AR.
- successful selection (puromycin resistance) and expansion of these CaP cells *in vitro*.
- demonstration of expression of expected exogenous gene products over control cells.
- produced AD- and CR-tumors *in vivo* driven by Src or Ack1.
- established baseline DHT-, Src- or Ack1-inducible AR-regulated gene assays at the level of qRT-PCR or ChIP (AR vs. Pol II).
- Successful production of AR-ChIP-seq and transcriptome-seq libraries and next-gen sequencing data
- Successful analysis of AR binding sequences induced by DHT, Src, Src/DHT, Ack1 or Ack1/DHT.
- Successful identification of three Src/Ack1-regulated gene signatures associated with CR-CaP.
- Validation of 20 genes for AR dependency and occupancy by ChIP and qRT-PCR.

REPORTABLE OUTCOMES

- isolation and validation of stable LNCaP/luc and LAPC-4/luc cells expressing CA-Src or -Ack1 plus WT or Y→F mutants of AR
- development of qRT-PCR and ChIP assays to assess DHT- vs. Src/Ack1-inducible AR-regulated genes.
- Data showing androgen-independent Src/Ack1-driven AR genomic binding sites
- Validated Src/Ack1 CR-driver gene signatures in comparison to published AR cistrome data from human CR-CaP samples.

CONCLUSION

We have successfully produced human CaP clones that express either activated Src or Ack1 in conjunction with WT or Y→F mutated AR, and showed that DHT-induced AR-regulated genes can be induced by Src or Ack1 in an AI fashion. Moreover, we showed that these AR-regulated genes still retain some androgen-inducibility after AR activation by Src or Ack1. CA-Src or Ack1 were sufficient to induce CR-CaP growth *in vivo*. We are generating AR-ChIP-seq and transcriptome-seq data in order to identify AR-engaged and differentially regulated genes induced by Src or Ack1 that might suggest pathways that drive CR-CaP growth in the absence of serum androgen levels.

REFERENCES

None

APPENDICES

None